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Technical Notes

Hydroquinone as a Buffer Additive for Suppression of Bubbles Formed by Electrochemical Oxidation of the CE Buffer at the Outlet Electrode in Capillary Electrophoresis/Electrospray Ionization-Mass Spectrometry

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Hydroquinone was found to suppress bubble formation at the outlet electrode of a sheathless capillary electrophoresis/electrospray ionization-mass spectrometer by replacing the oxidation of water ($2H_2O(l) \leftrightarrow O_2(g) + 4H^+ + 4e$) with that of more easily oxidized hydroquinone (hydroquinone $\leftrightarrow$ *p*-benzoquinone $+ 2H^+ + 2e$). Formation of *p*-benzoquinone replaces the formation of oxygen gas, effectively suppressing gas bubble formation. Several electrode materials, including platinum, gold-coated stainless steel, and stainless steel wires, were tested. However, hydroquinone suppressed bubbles only at the platinum electrode. Combination of the in-capillary electrode sheathless interface using platinum wire, hydroquinone as a buffer additive, and pressure programming at the inlet of the capillary electrophoresis provided a rugged high efficiency interface for analysis of protein digests using CE/ESI-MS.

The electrochemical nature of electrospray ionization (ESI) and capillary electrophoresis is well-known. It has been shown that the electrospray ion source is a controlled-current source and that the electrolytic nature of the ESI device is analogous to the controlled-current electrolysis carried out in a flow cell.[1−6] It is also well-known that electrolysis of the buffer due to the application of the separation voltage is a phenomenon accompanying electrophoresis.[7,8] Under capillary electrophoresis/electrospray ionization-mass spectrometry (CE/ESI-MS), where the CE outlet electrode also acts as the ESI electrode, these phenomena are combined. As a result, under commonly used CE/ESI-MS experimental parameters (aminopropylsilane-derivatized column, reverse polarity mode, 0.1% acetic acid buffer solution),[9−12] the CE outlet/ESI electrode acts as the anode at which the major oxidation reaction for aqueous buffer solutions is [typically] the electrochemical oxidation of water.[2] As a consequence of this oxidation reaction at the outlet electrode, the pH of the solution decreases, oxygen gas forms, and the electrode degrades due to electrochemical effects. While the pH change in the CE capillary[13] and in the electrospray ionization needle[6,14,15] and the electrochemical degradation of the CE outlet electrode[16] have been investigated, very little attention has been paid to the effect of bubble formation in CE/ESI-MS analysis.

In general, there are two different types of capillary electrophoresis-to-mass spectrometry interfaces using electrospray ionization: sheath−flow and sheathless.[11,17−23] In sheath−flow interfaces, the potential to the CE outlet electrode is provided through a conductive liquid that mixes with the CE buffer at or near the CE outlet. In sheathless interfaces, the potential to the CE outlet

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electrode is usually provided through a conductive metal. Bubble formation and/or degradation of the CE outlet/ESI electrode adversely affects CE/ESI-MS operation of both sheathless[24] and sheath−flow[25] interfaces that have direct metal/liquid contact. Their effects, however, are more pronounced in sheathless interfaces since the electrical connection to the CE outlet is usually provided through a thin conductive coating at the tip of the CE capillary outlet which can easily degrade. Moreover, bubbles formed at the point of contact between the CE outlet/ESI electrode and the CE buffer can easily interrupt the low (nanoliters/minute) buffer flow rates of the sheathless interface which in turn causes fluctuation of the CE and ESI currents. Nevertheless, because of the sensitivity advantage of sheathless over sheath−flow interfaces, it is desirable to develop long-lasting and stable sheathless interfaces. While the problem with quick degradation of the CE outlet/ESI electrode under sheathless conditions was recently addressed,[16,23] the issue of bubble formation still acts as a major obstacle to achieving long-lasting CE/ESI-MS operation with high separation efficiency. In this article, we address bubble formation and introduce a buffer additive for the suppression of bubbles to achieve high-performance sheathless CE/ESI-MS.

EXPERIMENTAL SECTION

Pt, gold-coated stainless steel, and stainless steel wires (Goodfellow, Berwyn, PA) were used for the fabrication of the in-capillary electrode;[16,23] however, no significant reduction of bubbles was observed for the latter two wires. Therefore, a 10-μm-diameter Pt wire was used with a 30-μm-i.d., 140-μm-o.d., 55-cm-long capillary, and a 25-μm-diameter Pt wire was used with a 50-μm-i.d., 150-μm-o.d., 70-cm-long capillary. Both capillaries (Polymicro Technology, Phoenix, AZ) were derivatized with aminopropylsilane (APS).[26] Unless otherwise mentioned, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Cytochrome *c* and hemoglobin S were digested following a previously reported procedure.[27] Digest solutions containing ~23 μM peptides were filtered and used without any pretreatment. Acetic acid buffer solutions (0.1% v/v) containing 0, 5, 10, 20, and 50 mM hydroquinone (HQ) were used. However, changing the molarity of the HQ from 10 to 50 mM produced similar results. Therefore, buffer containing 10 or 20 mM HQ was used.

A P/ACE System 2100 (Beckman Instruments, Fullerton, CA) CE instrument and a PerSeptive Biosystems Mariner (Framingham, MA) electrospray ionization time-of-flight mass spectrometer (ESI-TOFMS) were employed. The TOFMS was operated in the range of m/z 500−1500. Each data point on the electropherogram represents a total of 5000 acquisitions/0.5 s.

For all CE/ESI-MS experiments, the CE outlet/ESI electrode was maintained at ~2 kV while the CE inlet electrode was at −30 kV. The flow rate for the 30-μm-i.d., 55-cm-long capillary was ~0.1 μL/min, and the ESI current was 0.3−0.5 μA. The flow rate for the 50-μm-i.d., 70-cm-long capillary was 0.2−0.3 μL/min, and the ESI current was 0.8−1.0 μA.

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RESULTS AND DISCUSSION

Mechanism of Bubble Formation. Under commonly used CE/ESI-MS experimental parameters, the outlet electrode acts as the oxidative electrode (anode) at which the major oxidation reaction is the electrochemical oxidation of water according to the following reaction:[28]

$$2H_2O(l) \leftrightarrow O_2(g) + 4H^+ + 4e; \quad E^\circ_{red} = 1.229 \text{ V (vs SHE)}$$

Observation of the CE outlet of our in-capillary electrode sheathless interface under a magnifying glass reveals the formation of tiny gas bubbles at the outlet electrode. Because of the relatively high electroosmotic flow (EOF) of a freshly APS-derivatized CE column (approximately 200−300 nL/min for a 50-μm-i.d., 70-cm-long column), these small bubbles are swept out of the CE capillary column at a rapid rate and therefore have minimal effect on the CE or electrospray process. However, the separation efficiency of capillary electrophoresis under these high electroosmotic flows is not adequate for separation of complex biological mixtures such as protein digests.[24]

To achieve higher separation efficiencies, researchers usually condition their freshly derivatized CE capillaries by running CE buffer through them for several hours. Under this treatment, the APS coating of the inner wall apparently degrades, which leads to slower electroosmotic flow and higher separation efficiency. However, as columns age, reduction in EOF due to degradation of the derivatized CE inner wall increases the bubbles' size by lengthening their residence time in the vicinity of the electrode and inside the capillary. Since the degradation of the inner wall coating and formation of bigger bubbles is a gradual process, with time the CE performance also deteriorates. Eventually, the bubbles become large enough that they open the contact between the outlet electrode and the buffer solution, effectively halting the CE/ESI process.

To increase the useful life of the CE capillary, we recently developed pressure-assisted CE/ESI-MS with pressure-programming capability[24] that would augment the reduced EOF for optimum separation efficiencies. This technique, however, did not address gas production at the surface of the outlet/ESI electrode, which interrupts the current flow between the CE outlet/ESI electrode and the CE buffer. The weak coupling between this electrode and the buffer solution, in turn, causes fluctuation of the CE current and nonuniform pulsation of the spray. Two experimental consequences of this pulsation include increased background fluctuation (noisy electropherograms) and overlap of peaks with close migration times (low resolution) due to nonuniform accumulation of the CE buffer at the outlet of the CE capillary. Figure 1A shows the total ion electropherogram (TIE) of the background formed by CE/ESI-MS of 0.1% acetic acid buffer solution. Because a 3-month-old APS-derivatized column was used, no flow was present when a separation voltage of 32 kV was applied across the CE capillary. To establish the flow, a nitrogen gas pressure of ~2 psi (above atmosphere) was applied to the CE inlet 1 min after the separation voltage was turned on. However, as is shown in Figure 1A, the TIE was still very noisy.

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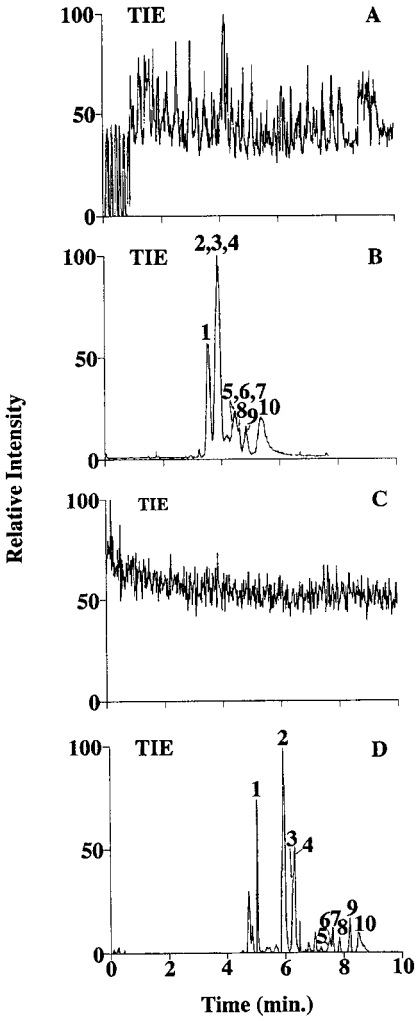

Figure 1. (A) TIE of the background ions generated under CE/ESI-MS using 10 mM acetic acid buffer solution. (B) TIE of the CE/ESI-MS analysis of a cytochrome *c* digest using 10 mM acetic acid buffer solution: 1, acetyl-GDVE; 2, EDLIAYLK; 3, TCQAPGFTYTDANK; 4, EETLMEYLENPK; 5, YIPGTK; 6, MIFAGIK; 7, MIFAGIK; 8, GITWK; 9, TGPNLHGLFGR; 10, CAQCHTVEK (heme). (C) TIE of the background ions generated under CE/ESI-MS using 10 mM acetic acid buffer solution containing 20 mM HQ additive. (D) TIE of the CE/ESI-MS analysis of a cytochrome *c* digest using 10 mM acetic acid buffer solution containing 20 mM HQ additive. The 50-μm-i.d. column was used. Approximately 30 fmol of each peptide was injected.

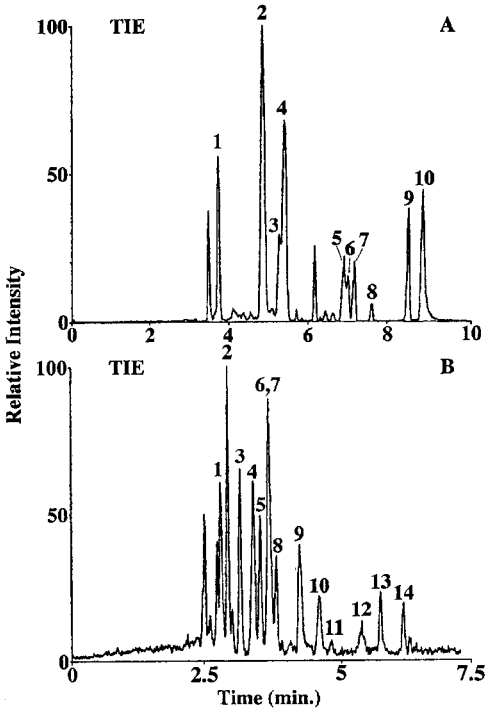

Figure 2. (A) TIE of CE/ESI-MS analysis of tryptic digests of cytochrome *c*. After peak 4 eluted, 3.5 psi pressure was applied to the inlet of the capillary and was ramped to 5.0 psi as peak 8 appeared: 1, acetyl-GDVE; 2, EDLIAYLK; 3, TCQAPGFTYTDANK; 4, EETLMEYLENPK; 5, YIPGTK; 6, MIFAGIK; 7, MIFAGIK; 8, GITWK; 9, TGPNLHGLFGR; 10, CAQCHTVEK (heme). Unmarked peaks are not identified. (B) TIE of CE/ESI-MS analysis of tryptic digests of hemoglobin S. A pressure of 2.0 psi was applied for the separations, and then the pressure was ramped to 3.5 psi after peak 13 eluted from the column: 1, FFESFGDLSTPDAVMGNPK; 2, VNVDEVGGEALGR; 3, EFTPPVQAAYPK; 4, FLASVSTVLTSK; 5, LLVVYPWTQR; 6, MFLSFPTTK; 7, VLGAFSDGLAHLDNLK; 8, SAVTALWGK; 9, VGAHAGEYGAEALER; 10, LHVDPENFR; 11, AAWGK; 12, VVAGVANALAHK; 13, TNVK; 14, VHLTPVEK. Unmarked peaks are not identified.

When the tryptic digest of cytochrome *c* was analyzed under these conditions (Figure 1B), most peaks overlapped.

Mechanism of Bubble Suppression by HQ. Electrochemists have used redox buffers to control the electrochemical potentials at the electrodes of the electrochemical cells.[29] Recently, organic additives were suggested as redox buffers for controlling the electrochemically induced pH change in the capillary.[8] Moreover, cesium iodide has been used as a redox buffer to control the pH change inside the electrospray needle.[6] The function of the redox buffer in these experiments was to hold the potential at the anode below that necessary to oxidize water and, therefore, to maintain the pH. A similar idea was used in this study to control the bubble formation inside the capillary of the CE/ESI-MS. However, under ESI/MS conditions, it is desirable to use a redox buffer whose oxidized and reduced forms do not compete with the analyte of interest for the available charge. Electrochemists have studied the electrochemical oxidation of aqueous solutions of HQ at platinum electrodes.[30,31] It was observed that once hydroquinone was added to water, it altered the electrochemical reaction at the electrode by replacing the oxidation of water with oxidation of more easily oxidized hydroquinone according to the following equation:[32]

$$\text{hydroquinone} \leftrightarrow p\text{-benzoquinone} + 2H^+ + 2e; \quad E^\circ_{\text{red}} = 0.699 \text{ V (vs SHE)}$$

Under this condition, the oxidation of hydroquinone replaces the electrochemical oxidation of water. The formation of water-soluble

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p-benzoquinone (2,5-cyclohexadiene-1,4-dione) replaces the formation of oxygen gas in the electrochemical oxidation of water, therefore leading to bubble reduction. Observation of the capillary outlet under a magnifying glass confirmed significant reduction of bubbles when HQ was added to the CE buffer. As a result, the CE/ESI-MS operation, which ceased under acetic acid buffer conditions, resumed without the need for external pressure (Figure 1C). Comparison between the background spectrum of panels A and C of Figure 1 revealed the presence of a low-intensity m/z 109 (protonated *p*-benzoquinone). However, the intensity of this signal was insignificant with respect to the other background signals that were present. This is an important characteristic of hydroquinone since neither the oxidized nor reduced forms contribute much gas-phase signal. This was confirmed by examining the effect of HQ on the sensitivity of detection for the tryptic digest of cytochrome *c* using the acetic acid buffer solution with (Figure 1D) and without (Figure 1B) the HQ additive. The areas under several ion electropherogram peaks of the two runs were examined and they were similar within an experimental error of ±5%. However, sharper peaks were obtained when the buffer contained HQ.

For analysis of protein digests, higher separation efficiencies have been reported by using narrower, APS-derivatized, conditioned columns in conjunction with pressure programming.[24] When a 30-μm-i.d. column, acetic acid solution containing 10 mM HQ, and the pressure-programming technique were used, most peptide fragments of cytochrome *c* (Figure 2A) and hemoglobin S (Figures 2B) were baseline separated. A significant enhancement of resolution was thus observed as a result of using HQ as a buffer additive compared with our previously reported CE/ESI-MS of the two digests.[23,24]

Without the addition of HQ to the CE buffer, bubble formation was observed with all three wires as the anode. In the case of the more reactive stainless steel, one might have expected bubble formation to be mitigated because of the competition between corrosion and water oxidation; however, stainless steel may also be inert since its surface is usually passivated with a layer of chromium oxide.[2,6] With addition of HQ to the CE buffer, the bubble reduction was only observed with platinum wire as the anode. This behavior could be attributed to the reduced potential of the Pt electrode in the presence of HQ as a redox buffer; however, adsorptive characteristics of HQ at the platinum surface[31] that can lower the ability of O_2 to nucleate into a bubble at the HQ-coated surface cannot be ruled out. More detailed studies of the mechanism of bubble suppression are underway.

Combination of the in-capillary electrode sheathless interface using platinum wire, HQ as a buffer additive, and pressure programming provides a rugged high-efficiency interface for analysis of peptide mixtures using CE/ESI-MS.

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